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14. ABSTRACT
Autophagy has emerged as a tumor cell survival mechanism in response to various stresses such as hypoxia and chemotherapy. As a result, there is rising interest in autophagy inhibition as an adjuvant cancer treatment. Given that carcinomas evolve with an inflammatory, desmoplastic stroma with significant numbers of cancer associated fibroblasts (CAFs), one can predict that autophagy is also induced in in these cells, allowing for their adaptation and function within tumor microenvironments. How autophagy inhibition in CAFs impacts tumorigenesis remains unclear. This proposal seeks to understand how stromal fibroblast specific ATG deletion effects mammary tumor progression, and seeks to determine the mechanisms by which this stromal deletion influences mammary epithelial cell fate and behavior. During the first year of this proposal, we generated a compound transgenic mouse model of mammary cancer (MMTV-PyMT) harboring genetic deletion of Atg12 in stromal fibroblasts using the fibroblast specific promoter FSP1 to drive Cre recombinase. We have characterized mammary tumor growth, metastasis and survival in these mice on mixed background. Our results suggest a trend toward reduced tumor burden and decreased metastasis in mice with autophagy deletion in stromal fibroblasts. We are currently obtaining larger sized cohorts for these studies. Also, this year, we have optimized the isolation and infection of both mouse mammary fibroblasts and CAFs for in vitro and in vivo analyses. In the upcoming year, we will investigate the mechanism by which autophagy deletion in stromal fibroblasts reduces mammary tumor progression.

15. SUBJECT TERMS
fibroblast, cancer associated fibroblast (CAF), autophagy, Atg12, FSP-Cre, desmoplasia, breast cancer, collagen

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INTRODUCTION

Breast cancer is a heterogeneous, multi-factorial disease of aberrant breast development whose etiology relies upon microenvironmental changes within the tissue. Such changes involve the appearance of α-smooth muscle actin positive (αSMA+) fibroblasts (referred to as "cancer associated fibroblasts", or CAFs), recruitment of various immune cells (macrophages, T cells, B cells, T regulatory cells), and enhanced type I collagen deposition [1]. Within the last decade, a plethora of evidence demonstrates the importance of this inflammatory and desmoplastic stromal response to the initiation and progression of breast cancer [2]. In particular, CAFs are the major cell type responsible for tumor desmoplasia, as these cells secrete abundant levels of type I collagen which alters the stiffness and compliance of the tumor associated extra-cellular matrix (ECM) [3]. ECM stiffening, as a result of CAF-mediated collagen deposition, enhances integrin and PI3K signaling, both of which support mammary tumor initiation and progression [4]. While the functional contribution of CAFs to breast cancer progression is well documented, the molecular mechanisms and cellular pathways that govern CAF behavior remain poorly understood. Importantly, CAFs are exposed to the same hypoxic and nutrient deprived microenvironments as tumor cells [5]; thus, they most likely upregulate stress response pathways in order to survive under these conditions. A commonly upregulated stress response pathway is autophagy, an evolutionarily conserved cellular stress response pathway that serves to remove damaged proteins and organelles and prevent cellular toxicity. The process involves the formation of a double membrane organelle, the autophagosome, which sequesters cytoplasmic contents and fuses with the lysosome for degradation and metabolite recycling [6, 7]. Several cancer-relevant stresses induce autophagy, including nutrient deprivation, hypoxia, the unfolded protein response, mechanical stress and chemotherapy [6, 8]. Because autophagy most often functions as a cellular survival mechanism, autophagy inhibitors in conjunction with chemotherapy could hold tremendous promise in the eradication of breast cancer [9-12]. Currently, the autophagy inhibitor hydroxychloroquine (HCQ) is in phase I and II clinical trials as an adjuvant therapy for the treatment of metastatic breast cancer [13, 14]. **However, it remains largely unknown how autophagy inhibition alters the behavior of stromal cell types, and how this ultimately impacts the growth and survival of breast tumor cells.** By utilizing mice harboring floxed alleles of essential autophagy genes, I will determine how tissue specific deletion of autophagy in stromal fibroblasts impacts mammary tumor development.

KEYWORDS

fibroblast, cancer associated fibroblast (CAF), autophagy, Atg12, FSP-Cre, desmoplasia, breast cancer, collagen

ACCOMPLISHMENTS

In accordance with the approved Statement of Work for this proposal, 4 subtasks were assigned to Year 1. We have made substantial progress towards completing these tasks and have a stronger understanding of how autophagy inhibition in fibroblasts affects mammary tumor development, progression, and metastasis.

Task 1: To determine the effects of stromal fibroblast specific atg deletion on mammary tumor progression.

Subtask 1a: Obtain ACURO approval (months 1-2).

We have successfully obtained ACURO approval in accordance with Debnath Animal Protocol # AN107285-01.

Subtask 1b: Generate Atg12fl/fl;RFP+;FSP-Cre;MMTV-PyMT, Atg5fl/fl;RFP+;FSP-Cre;MMTV-PyMT, and control RFP+;FSP-Cre;MMTV-PyMT mice (months 2-18).

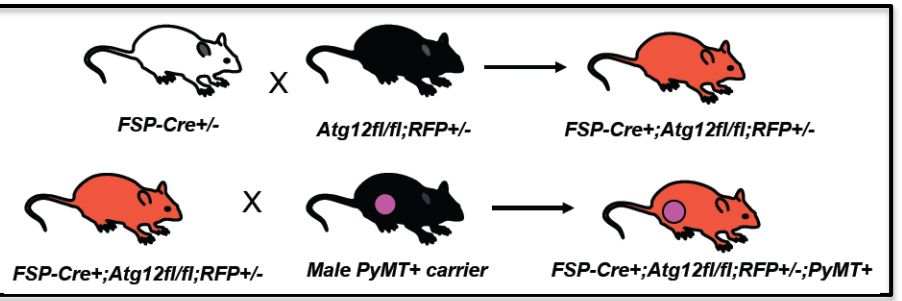
Using the breeding strategy outlined in Figure 1, we have successfully generated these quadruple transgenic mice. We genotyped these mice by isolating genomic DNA from mouse tails and performing PCR for the floxed Atg12 allele, RFP allele, FSP-Cre allele and PyMT allele (Fig. 2).

Moreover, we confirmed the presence of the effectively recombined, null allele at the Atg12 locus by PCR (Fig. 3A), demonstrating recombination only in mice carrying the FSP-Cre allele. To further corroborate these PCR results, we digested mammary glands from *Atg12fl/fl;RFP+/RFP+;FSP-Cre+* mice and performed fluorescence activated cell sorting (FACS) on the digested tissue. Two populations were present in the digested tissue: an RFP+ population and RFP- one (Fig. 3B). As a negative control, digested mammary glands from *Atg12fl/fl;RFP+/RFP+;FSP-Cre−* mice did not show any RFP expression (Fig. 3C). Combining the RFP

sorting strategy with an antibody against EpCAM (a marker of epithelial cells) showed that the majority of RFP expression is enriched in the RFP+/EpCAM- fraction (Fig. 3D). These data suggest that FSP is not expressed in the mammary epithelium and Cre recombinase expression is limited to the stromal cells as expected.

We microsatellite sequenced these mice to determine their C57B/6 purity and estimate the amount of time it would take to backcross these mice to a pure B/6 background for transplant purposes. We chose 2 mice of each genotype and averaged the purity. Our data suggest that these mice are ~74% C57B/6 (summarized in Table 2; see Appendix) and this would take at least 5 backcrosses to reach the purity level we would need for transplantation. Given the complex allelic combination and number of backcrosses required, we have chosen to characterize these mice on their mixed background and perform transplant experiments without *FSP-Cre*.

Figure 1. Generation of quadruple transgenic mice. Breeding scheme to generate quadruple transgenic mice. *FSP-Cre* mice were obtained on FvB background, and bred to our C57B/6 pure *Atg12fl/fl;RFP+/-* mice. These mice (red) were then bred to a male *PyMT+* carrier to initiate mammary tumorigenesis.



***Subtask 1c:* Determine the latency period for the onset of primary tumor formation and metastasis for transgenic mice generated in Subtask 1b. At selected time points, 10 mice from each experimental cohort will be sacrificed and evaluated for characteristics of histopathologic progression from primary to metastatic disease (invasion, tumor cell proliferation/cell death, tumor burden, inflammatory cell recruitment, angiogenesis, and development/latency to metastasis). (months 6-30)**

We have monitored tumor development of the *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* (n=13) and *Atg12fl/fl;RFP+;FSP-Cre−;PyMT+* (n=11) mice over 121 days. While tumor volume at endpoint does not appear to be significant (Fig. 4A), we are currently investigating more mice (ideally n=20 per cohort) to see if metastatic burden differs at endpoint between *Cre+* and *Cre−* cohorts (Fig. 4B). In addition, we have analyzed earlier time points for differences in hyperplasia/disease progression (Fig. 5). At 85 days, the extent of hyperplasia between *Cre-* and *Cre+* mice is not dramatically different among all mice examined thus far. Thus, we are currently investigating the status of mammary hyperplasia at 15 and 30 days of age (data in progress; not shown).

***Subtask 1d:* Optimize the harvest and propagation of mouse cancer associated fibroblasts (CAFs) from the mammary glands of Atg12fl/fl;RFP+;FSP-Cre+;PyMT+, Atg5fl/fl;RFP+;FSP-Cre+;PyMT+, and control RFP+;FSP-Cre+;PyMT+ mice. (months 6-12)**

We have encountered a technical issue while performing this subtask as described. We first attempted to optimize the isolation and culture of MMFs from *Atg12fl/fl;RFP+;FSP-Cre+* mammary glands before utilizing our *Atg12fl/fl;RFP+;FSP-Cre+*;*PyMT+* mice. We wanted to prioritize these quadruple transgenic mice to build sufficient numbers for the tumor development and metastasis studies described in Figures 4 and 5. Thus, utilizing our previously established MMF isolation protocol (see Appendix), we attempted to generate cultures of *Atg12fl/+;RFP+;FSP-Cre+* (autophagy competent) and *Atg12fl/fl;RFP+;FSP-Cre+* (autophagy incompetent) MMFs that we could then use for generating lysate and interrogation by Western blot (Fig. 7). However, our data suggest that the autophagy incompetent MMFs (from *Atg12fl/fl;RFP+;FSP-Cre+* mammary glands*)* do not grow out in culture. We have been unable to detect effective recombination at the Atg12 locus or ablation of autophagy from cell cultures *in vitro* (despite evidence for recombination *in vivo*, Figs. 2 and 3). There is likely selectivity in culture for the MMFs that remain autophagy competent. In summary, using the germ-line expression of *FSP-Cre* to isolate autophagy deficient MMFs for *in vitro* studies will be difficult.

To circumvent this issue, we utilized our *Atg12fl/fl;RFP+;PyMT+* mice on a pure B/6 background to optimize the isolation of CAFs from their tumor-burdened mammary glands (for protocol, see Appendix). We sorted these CAFs from digested tumors using the sorting strategy outlined in Figure 7A. Enriching for PDGFRα+ cells and depleting F4/80+ cells (macrophages), we obtained a significant minority of the tumor cell population (~1%). These sorted cells ("CAFs") were then plated on type I collagen (optimal culture conditions for primary mouse fibroblasts). However, these cells failed to proliferate which rendered insufficient cell

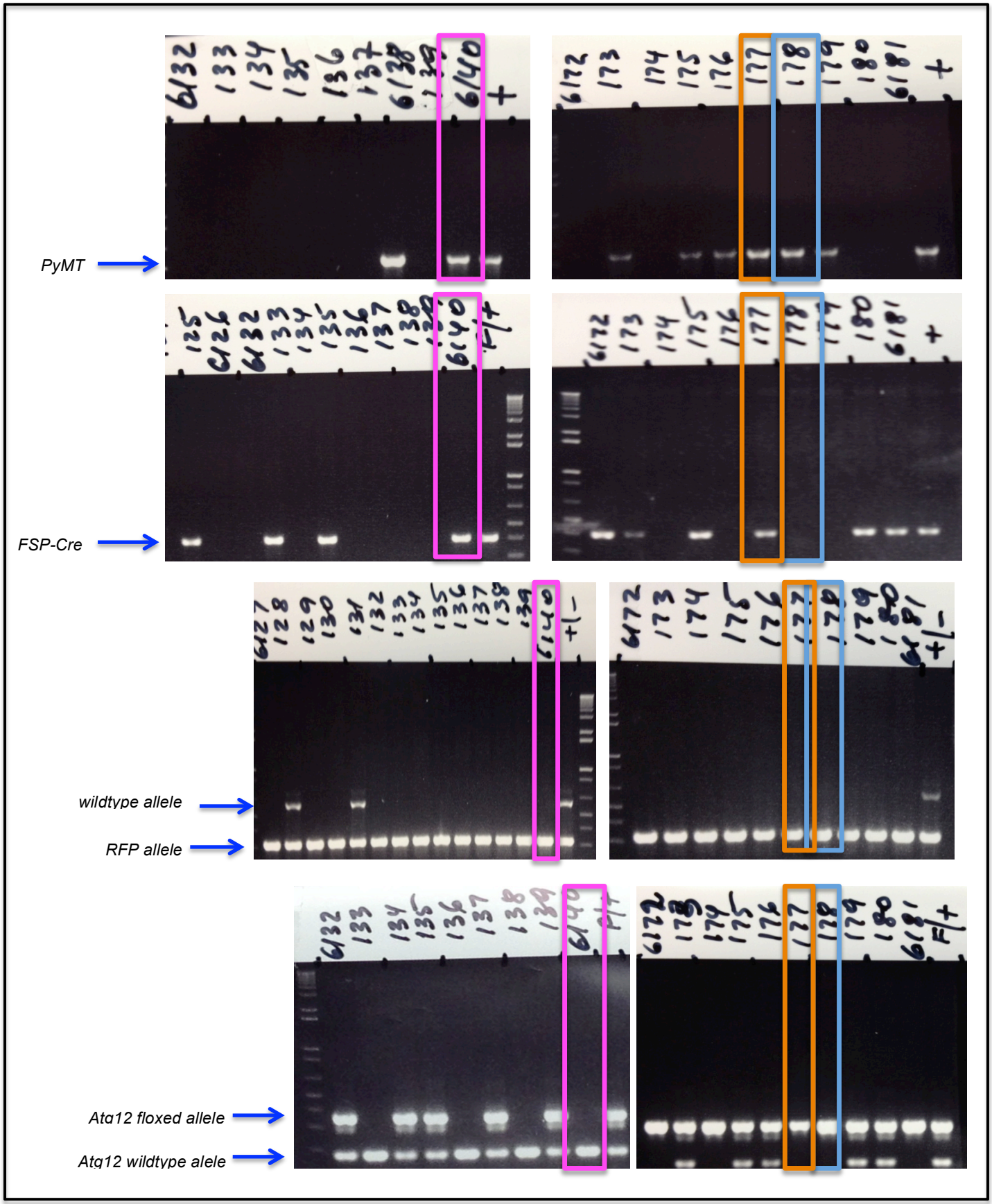


Figure 2: Genotyping results for generation of quadruple transgenic mice described in Fig. 1

numbers for further experimentation. As a result, we immortalized these CAFs by infecting them with a

Female Mouse #	Genotype
6140	*RFP+/RFP+;FSP-Cre+;PyMT+*
6177	*Atg12fl/fl;RFP+/RFP+;FSP-Cre+;PyMT+*
6178	*Atg12fl/fl;RFP+/RFP+;FSP-Cre−;PyMT+*

Table 1. Genotypes of mice as determined from PCR products in Figure 2 above.

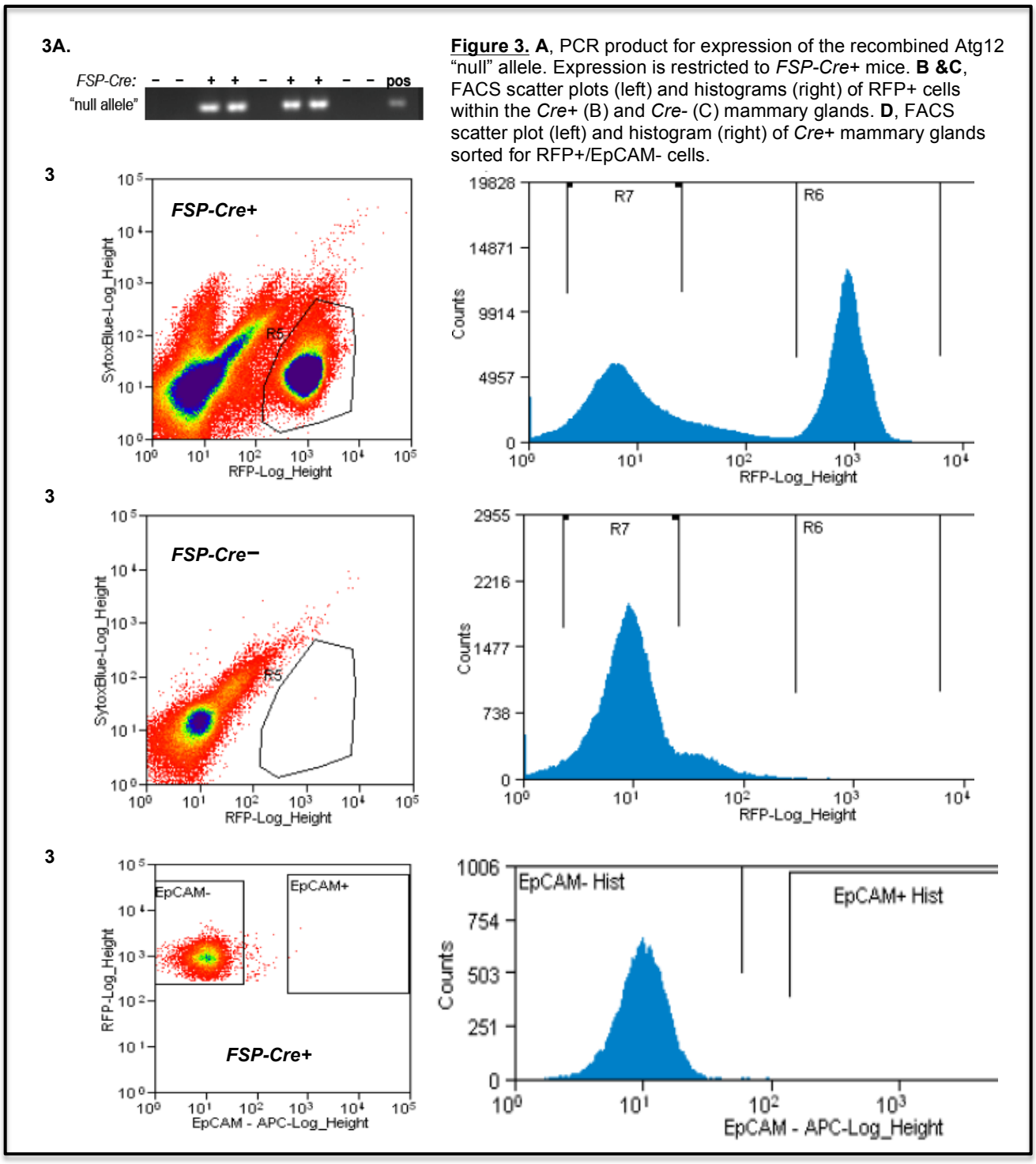


Figure 3. A, PCR product for expression of the recombined Atg12 "null" allele. Expression is restricted to *FSP-Cre+* mice. **B &C**, FACS scatter plots (left) and histograms (right) of RFP+ cells within the *Cre+* (B) and *Cre-* (C) mammary glands. **D**, FACS scatter plot (left) and histogram (right) of *Cre+* mammary glands sorted for RFP+/EpCAM- cells.

retrovirus expressing SV40 Large T Antigen. After selection, an immortalized population of fibroblasts emerged, as evidence by expression of the fibroblast markers αSMA, vimentin and FSP (Fig. 7B), and lack of

Genotype	Average Purity
Atg12fl/fl;RFP+/RFP+;FSP-Cre+;PyMT+	75.5%
Atg12fl/fl;RFP+/RFP+;FSP-Cre−;PyMT+	73.4%

Table 2. Calculated average C57B/6 purity of mice subjected to microsatellite sequencing.

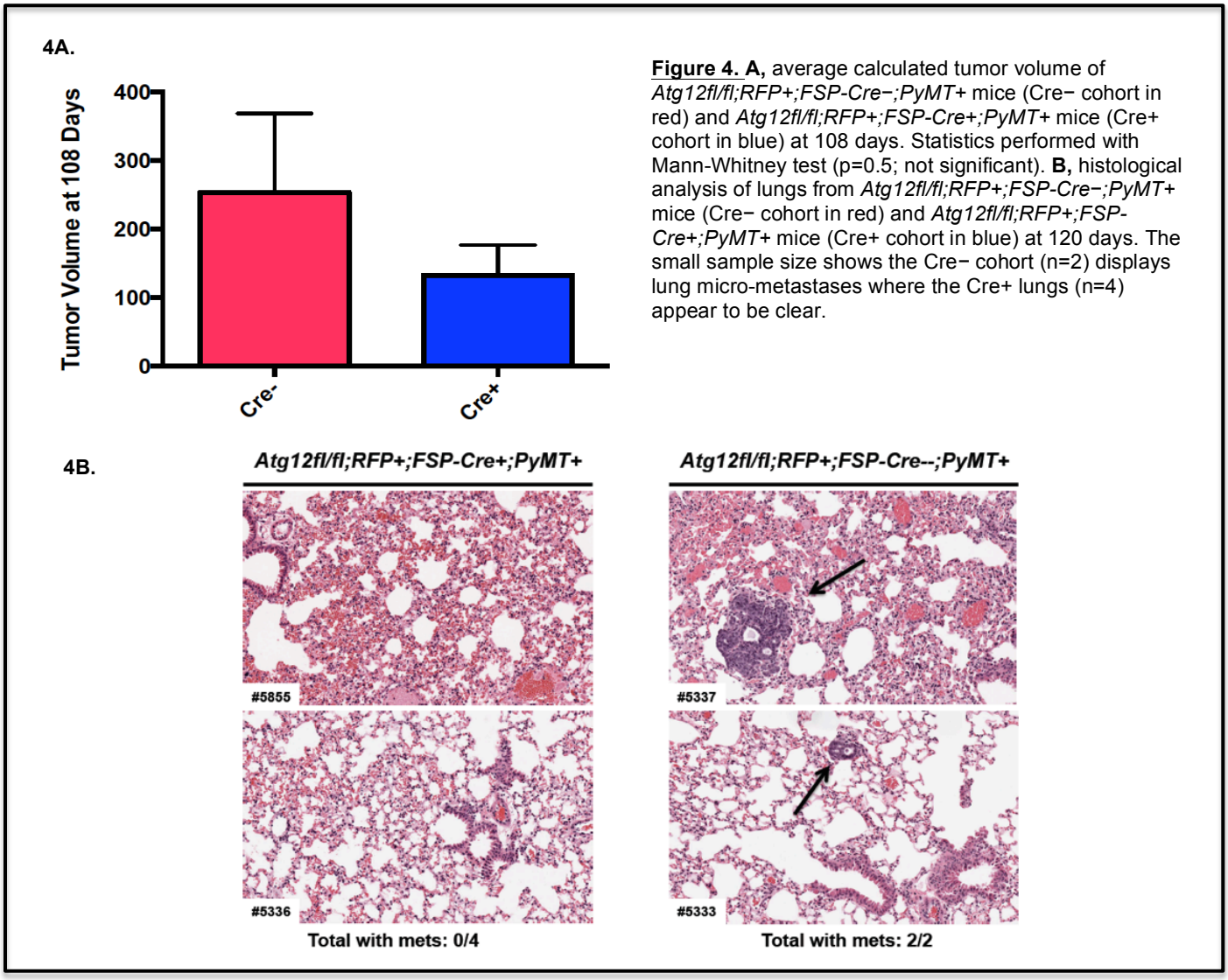


Figure 4. A, average calculated tumor volume of *Atg12fl/fl;RFP+;FSP-Cre−;PyMT+* mice (Cre− cohort in red) and *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* mice (Cre+ cohort in blue) at 108 days. Statistics performed with Mann-Whitney test (p=0.5; not significant). **B,** histological analysis of lungs from *Atg12fl/fl;RFP+;FSP-Cre−;PyMT+* mice (Cre− cohort in red) and *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* mice (Cre+ cohort in blue) at 120 days. The small sample size shows the Cre− cohort (n=2) displays lung micro-metastases where the Cre+ lungs (n=4) appear to be clear.

expression of the mammary epithelial cytokeratins CK14 (Fig. 7B) and CK8 (data not shown). Moreover, lysate obtained from these immortalized CAFs shows significant expression of FSP protein as compared to lysate obtained from primary PyMT tumor cells (Fig. 7C), further corroborating our choice of using FSP as a fibroblast specific Cre driver. Lastly, these immortalized CAFs were then infected with adenovirus expressing Cre recombinase or an empty vector. After 7 days, sufficient recombination at the Atg12 locus was evident as shown by Western blot (Fig. 7D), rendering the cells autophagy incompetent, as shown by the lack of LC3-II expression (lower band, Fig. 7D). These cells also effectively turned on RFP expression after exposure to Cre recombinase (data not shown). Thus, our results indicate we have optimized a CAF isolation method from PyMT tumors that allows us to effectively delete autophagy in these cells. We are currently using this FACS strategy to obtain CAFs from our *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* mice for *in vivo* co-mixing experiments (as proposed in Task 2, year 2).

Subtask 1f. Perform syngeneic transplants with autophagy competent (RFP+;FSP-Cre) and autophagy incompetent (Atg12fl/fl;RFP+;FSP-Cre+ and Atg5fl/fl;RFP+;FSP-Cre+) mouse mammary fibroblasts

(MMFs) into C57B/6 hosts, followed by inoculation with tumor cells 3 weeks following engraftment. (months 18-36)

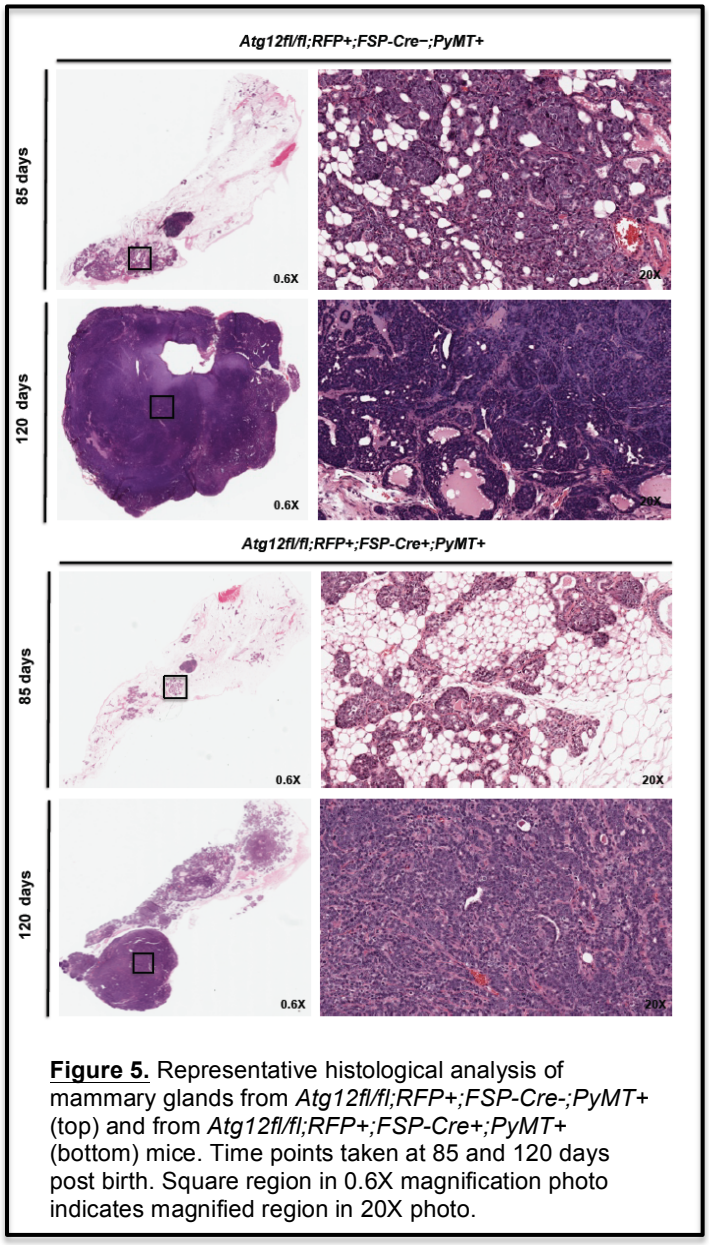


Figure 5. Representative histological analysis of mammary glands from *Atg12fl/fl;RFP+;FSP-Cre-;PyMT+* (top) and from *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* (bottom) mice. Time points taken at 85 and 120 days post birth. Square region in 0.6X magnification photo indicates magnified region in 20X photo.

These studies are planned for the upcoming year. However, we potentially see two technical issues that will affect our approach for this subtask. First, as discussed above, we have failed to obtain MMF cultures from *Atg12fl/+;RFP+;FSP-Cre+* (autophagy competent) and *Atg12fl/fl;RFP+;FSP-Cre+* (autophagy incompetent) mammary glands. Our data suggest that the autophagy incompetent MMFs (from *Atg12fl/fl;RFP+;FSP-Cre+* mammary glands) do not grow out in culture (Fig. 6). Without pure, proliferating cultures of these MMFs, we cannot commence these transplant experiments. Second, syngeneic transplants cannot be completed unless we backcross these mice to pure C57B/6 background. Given the results of the microsatellite sequencing (summarized in Table 2), we suspect that we would have to go through at least 4 generations of backcrossing prior to commencing these experiments. We can circumvent the requirement for backcrossing by performing these experiments using an *ex vivo* Cre-mediated deletion method (see MMF isolation and Infection Protocol in Appendix). Specifically, we can isolate MMFs from either *Atg12fl/fl;RFP+* or *RFP+* (control) mice (already established on pure C57B/6 background) and transduce these cells with an adenovirus expressing Cre recombinase. We can clear the mammary epithelium from C57B/6 hosts and injected these cells into the cleared fat pad. We have already optimized the isolation and autophagy deletion in these cells. Furthermore, we have confirmed that both of these MMFs efficiently engraft into the cleared fat pad of the host mouse (data not shown).

Subtask 1g. Determine the latency period for the onset of primary tumor formation and metastasis for recipient mice generated in (f). At selected time points, 10 mice from each experimental cohort will be sacrificed and evaluated for characteristics of histopathologic progression from primary to metastatic disease (invasion, tumor cell proliferation/cell death, tumor burden, inflammatory cell recruitment, angiogenesis, and development/latency to metastasis). (months 24-36)

These studies are planned for the upcoming year.

Task 2: To determine if cytokines secreted by autophagy deficient FSP-Cre+ stromal fibroblasts influences mammary epithelial cell fate and behavior.

Subtask 2a. Use conditioned medium from cell populations isolated in Task (1d) to assess tumorgenicity of PyMT tumor cells* in vitro *using a "tumorsphere assay." (months 12-30).

These studies are planned for the upcoming year.

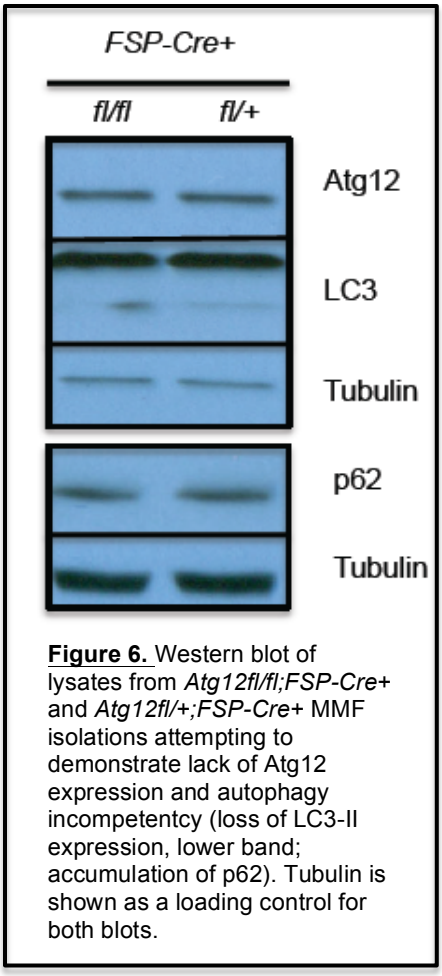


Figure 6. Western blot of lysates from *Atg12fl/fl;FSP-Cre+* and *Atg12fl/+;FSP-Cre+* MMF isolations attempting to demonstrate lack of Atg12 expression and autophagy incompetentcy (loss of LC3-II expression, lower band; accumulation of p62). Tubulin is shown as a loading control for both blots.

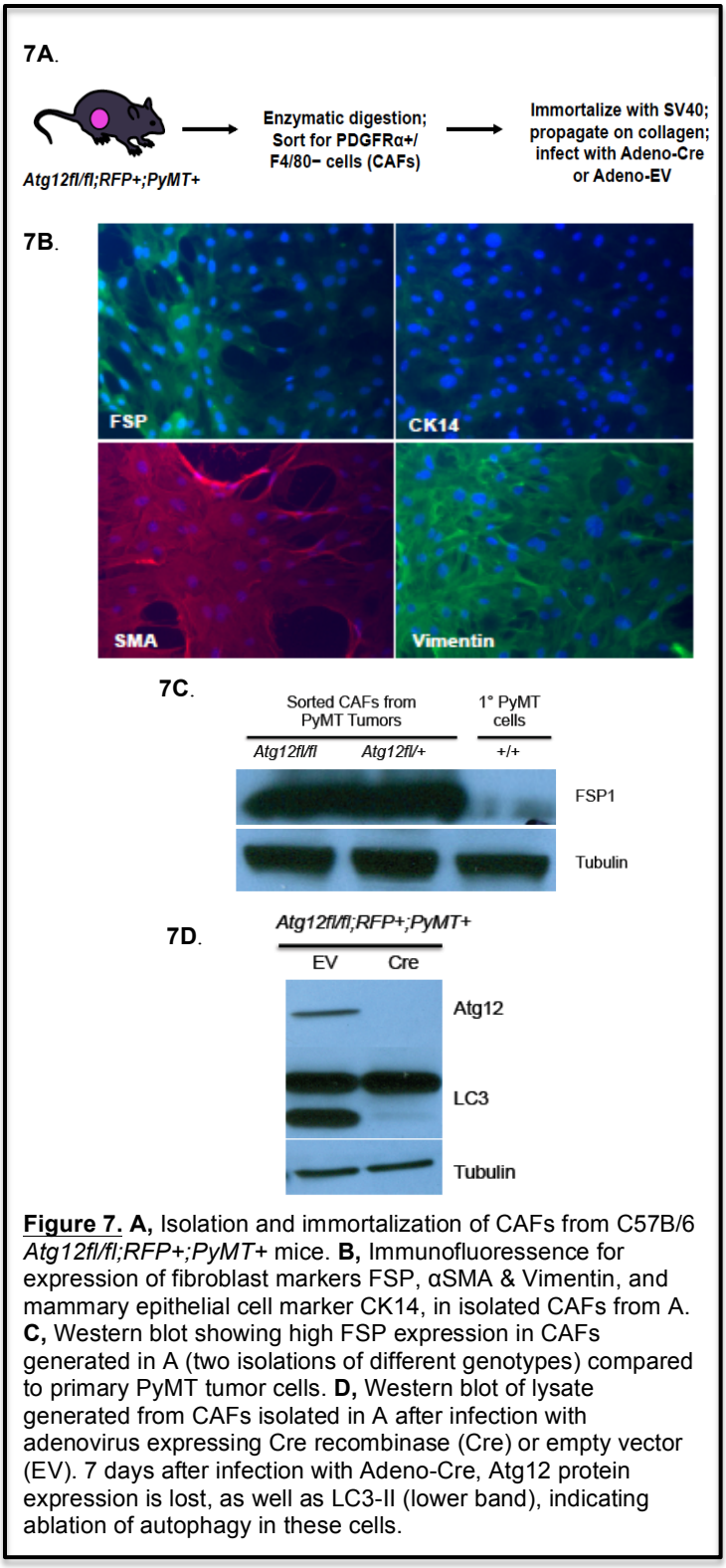


Figure 7. A, Isolation and immortalization of CAFs from C57B/6 *Atg12fl/fl;RFP+;PyMT+* mice. **B,** Immunofluoressence for expression of fibroblast markers FSP, αSMA & Vimentin, and mammary epithelial cell marker CK14, in isolated CAFs from A. **C,** Western blot showing high FSP expression in CAFs generated in A (two isolations of different genotypes) compared to primary PyMT tumor cells. **D,** Western blot of lysate generated from CAFs isolated in A after infection with adenovirus expressing Cre recombinase (Cre) or empty vector (EV). 7 days after infection with Adeno-Cre, Atg12 protein expression is lost, as well as LC3-II (lower band), indicating ablation of autophagy in these cells.

Subtask 2b.
Use conditioned medium from cell populations isolated in Task (1d) to assess invasive properties of PyMT tumor cells using a "scratch wound assay." (months 12-30).

These studies are planned for the upcoming year.

Subtask 2c. Establish three-dimensional co-cultures using autophagy competent or incompetent CAFs isolated in Task (1d) with PyMT tumor cells and evaluate how autophagy inhibition in CAFs effects invasive behavior, basement membrane deposition, cell-cell junction integrity, mammary differentiation, proliferation and apoptosis. (months 12-36)

These studies are planned for the upcoming year.

Subtask 2d. Obtain conditioned medium from cell populations isolated in Task (1d) to utilize for an unbiased cytokine array analysis, and validate potential candidates with mouse specific ELISAs. (months 24-30)

These studies are planned for the upcoming year.

Subtask 2e. ***Cytokines of interest from the results in Task (2d) will be interrogated for their functional contribution to tumorsphere formation, proliferation, survival, and apoptosis. (months 24-36)***

These studies are planned for the upcoming year.

Subtask 2f. ***Determine the functional consequence of differential cytokine secretion between autophagy competent and incompetent mouse CAFs by generating shRNAs against potential cytokine candidates determined in Task (2d) and knocking down their expression in mouse CAFs. After selection, these CAFs will be functionally interrogated by co-mixing with PyMT tumor cells and subsequent injection into the MFPs of syngeneic recipient mice. I will use the same ratio of fibroblasts to PyMT cells as described in Task (1e). Tumor growth will be monitored over time as compared to mouse CAFs infected with a non-targeting shRNA control, and compared to injection of PyMT tumor cells alone. I anticipate 30 donor mice per cohort will be used for MMF and PyMT cell isolation, and 30 host recipient animals per cohort will be used for MFP injection. (months 18-36)***

These studies are planned for the upcoming year.

SUMMARY OF KEY RESEARCH ACCOMPLISHMENTS

1. We have successfully generated the following cohorts of mice: *Atg12fl/fl;RFP+/RFP+;FSP-Cre–;PyMT+* (autophagy competent); *Atg12fl/fl;RFP+/RFP+;FSP-Cre+;PyMT+* (autophagy incompetent)*; RFP+/RFP+;FSP-Cre+;PyMT+* (autophagy competent).

2. We have generated evidence (using PCR and FACS analysis) to suggest efficient recombination at the *Atg12* and *RFP* loci *in vivo.*

3. We have begun to monitor tumor initiation, progression and metastasis in these mouse cohorts. Ideally, we would like n=20 mice per cohort for statistical analysis.

4. We have begun to monitor the onset of mammary hyperplasia in these mouse cohorts. Our preliminary data suggest differences in the extent of hyperplasia between *Cre+* and *Cre–* cohorts might be more apparent at early time points (15 or 30 days post birth).

5. We have successfully optimized the isolation of CAFs from *Atg12fl/fl;RFP+;PyMT+* mammary tumors and efficiently deleted autophagy in these cells.

6. We have successfully engrafted autophagy competent or incompetent MMFs into the cleared fat pad of C57B/6 hosts.

TRAINING AND PROFESSIONAL DEVELOPMENT

1. *Poster presentation*, Gordon Conference in Mammary Gland Biology
"Dissecting the Functions of Autophagy in Breast Cancer Associated Fibroblasts"
Lucca, Italy; June 8-13, 2014

2. *Oral presentation*, "Research in Progress" Postdoctoral Seminar Series
"Dissecting the Functions of Autophagy in Breast Cancer Associated Fibroblasts"
University of California, San Francisco; May 30, 2014

DISSEMINATED RESULTS

Nothing to report.

IMPACT

Breast cancer is a disease of aberrant breast development whose etiology relies upon microenvironmental changes within the tissue. Such changes involve the appearance of α-smooth muscle actin positive (αSMA+) fibroblasts (CAFs), recruitment of various immune cells (macrophages, T cells, B cells, T regulatory cells), and enhanced type I collagen deposition. Within the last decade, a plethora of evidence demonstrates the importance of this inflammatory and desmoplastic stromal response to the initiation and progression of breast cancer. Despite this, the majority of cancer therapeutics fail to address this stromal response, largely exploiting the molecular mechanisms and genomic instability of breast cancer cells. Stromal gene expression data can predict patient response to pre-operative chemotherapy and has been implicated in breast cancer recurrence and metastatic disease. CAFs in particular serve as attractive drug targets in breast cancer, given their genomic stability and abundance in tumor associated stroma. However, the molecular mechanisms and cellular pathways that govern CAF behavior must be characterized before they can serve as potential targets for breast cancer therapeutics.

Importantly, CAFs are exposed to the same hypoxic and nutrient deprived microenvironments as tumor cells, and they likely upregulate stress response pathways in order to survive under these conditions. A commonly upregulated stress response pathway is autophagy, an evolutionarily conserved cellular stress response pathway that serves to remove damaged proteins and organelles and prevent cellular toxicity. Several cancer-relevant stresses induce autophagy, including nutrient deprivation, hypoxia, mechanical stress and chemotherapy. It remains unknown whether CAFs upregulate autophagy in order to survive in the tumor microenvironment.

Remarkably, the autophagy inhibitor hydroxychloroquine (HCQ) is already in phase I and II clinical trials as an adjuvant therapy for the treatment of metastatic breast cancer. However, it remains largely unknown how autophagy inhibition alters the behavior of stromal cell types, and how this ultimately impacts the growth and survival of breast tumor cells. *The studies outlined in this fellowship will provide essential information on whether autophagy inhibitors used clinically to treat breast cancer harbor stromal side effects that either work in unison or antagonistically with regression of tumor growth***.**

CHANGES/PROBLEMS

1. We are currently evaluating the tumor growth kinetics of the *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* mice while on mixed background. Given the results of our microsatellite sequencing, we anticipate that backcrossing these compound transgenic mice onto a pure C57B/6 background will require an extended length of time. Accordingly, we will focus on the analysis from the mixed background animals over the upcoming year.

2. We have not yet validated inhibition of autophagy in the *Atg12fl/fl;RFP+;FSP-Cre+;PyMT+* mice *in vivo.* These data have been hindered by the inability to obtain sufficient numbers of CAFs from these mice without culturing them beforehand. Our goal over the upcoming year is to scale up the number of animals to obtain sufficient numbers of CAFs directly from the mammary tumors of these mice, in order to show autophagy inhibition by western blotting.

3. We have not yet optimized the isolation and growth of *Atg12fl/fl;RFP+;FSP-Cre+* and *RFP+;FSP-Cre+* MMFs *in vitro.* We can circumvent this problem by performing an *ex vivo,* Cre-mediated deletion of Atg12 in MMFs isolated from *Atg12fl/fl;RFP+* and *RFP+* mammary glands.

4. We cannot perform syngeneic transplants with MMFs isolated from the *Atg12fl/fl;RFP+;FSP-Cre+* and *RFP+;FSP-Cre+* mice because of their failure to grow out in vitro (see #3) and their mixed genetic background (see #1). We can potentially overcome this barrier in the upcoming years by transplanting *Atg12fl/fl;RPF+* and *RFP+* MMFs (already on a C57B/6 pure background) and utilizing an *ex vivo* Cre mediated deletion method.

PRODUCTS

Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Fanya Rostker	Florie Mar
Project Role:	Animal Technician	Graduate Student
Nearest person month worked:	5	2
Contribution to Project:	Ms. Rostker has performed work in the area of subtask 1b and 1c.	Ms. Mar has performed work in the area of subtasks 1c, 1d, and 1e.
Funding Support:	B.C.R.P. Innovator Scholar Concept Award (to Jayanta Debnath) and B.C.R.P. Era of Hope Scholar Award (to Jayanta Debnath)	National Science Foundation Fellowship Award (to Florie Mar)

APPENDIX I. Microsatellite sequencing data for FSP-Cre mice

Marker	Chr	Pos	4713	5337	5332	5335
D1Mit68	01	5.5	B6 FVB	B6	B6 FVB	B6 FVB
D1Mit171	01	18.6	B6 FVB	B6	B6	B6 FVB
D1Mit303	01	32.8	B6	B6	B6	B6
D1Mit132_G	01	43.7	B6	B6	B6	B6
D1Mit60	01	59	B6	B6	B6 FVB	B6
D1Mit102	01	75.4	B6	B6	B6 FVB	B6
D1Mit36	01	91.8	B6	B6 FVB	B6 FVB	B6
D1Mit221	01	104.9	B6	B6 FVB	B6	B6
D2Mit80	02	12	B6 FVB	FVB	B6 FVB	B6 FVB
D2Mit296	02	23	B6 FVB	B6 FVB	B6 FVB	B6 FVB
D2Mit182	02	38.3	B6	B6	B6	B6
D2Mit75	02	48.1	B6 FVB	B6	B6	B6 FVB
D2Mit304	02	59	B6 FVB	B6	B6	B6 FVB
D2Mit285	02	72.1	B6 FVB	B6	B6	B6 FVB
D2Mit343	02	84.2	B6	B6	B6 FVB	B6 FVB
D3Mit60	03	0	B6	B6	B6	B6
D3Mit46	03	12	B6	B6	B6	B6
D3Mit25	03	21.9	B6	B6	B6 FVB	B6 FVB
D3Mit57	03	40.4	B6	B6	B6 FVB	B6 FVB
D3Mit84	03	50.3	B6	B6	B6 FVB	B6 FVB
D3Mit19	03	66.7	B6 FVB	B6 FVB	B6 FVB	FVB
D4Mit104	04	3.3	B6	B6	B6	B6
D4Mit171	04	10.9	B6	B6	B6	B6
D4Mit178	04	30.6	B6	B6	B6 FVB	B6
D4Mit155	04	48.1	B6	B6	B6 FVB	B6
D4Mit204	04	61.2	B6 FVB	B6 FVB	FVB	B6
D4Mit234	04	71	B6 FVB	B6 FVB	FVB	B6
D4Mit256_G	04	82	B6 FVB	B6 FVB	B6 FVB	B6
D5Mit343	05	0	B6 FVB	B6	B6 FVB	B6 FVB
D5Mit267	05	16.4	B6	B6	B6	B6
D5Mit239	05	40.4	B6	B6 FVB	B6 FVB	B6
D5Mit136	05	53.6	B6	B6 FVB	B6 FVB	B6
D5Mit221	05	66.7	B6 FVB	B6	B6	B6 FVB
D5Mit169	05	82	B6 FVB	B6 FVB	B6	B6 FVB
D6Mit86	06	0	B6 FVB	B6	B6 FVB	B6 FVB
D6Mit224	06	10.9	B6 FVB	FVB	B6 FVB	B6 FVB
D6Mit188	06	21.9	B6 FVB	FVB	B6 FVB	B6 FVB
D6Mit31	06	31.7	B6 FVB	FVB	B6 FVB	FVB
D6Mit105	06	41.5	B6 FVB	FVB	B6 FVB	FVB
D6Mit14	06	63.4	B6	B6	B6	B6
D7Mit76	07	3.3	B6 FVB	FVB	B6 FVB	B6
D7Mit69	07	20.8	B6	B6 FVB	B6 FVB	B6
D7Mit220	07	38.3	B6	B6	B6	B6
D7Mit44	07	50.3	B6	B6	B6	B6
D8Mit223	08	8.7	B6	B6 FVB	B6	B6

D8Mit205	08	31.7	B6	B6	B6	B6
D8Mit183	08	47	B6	B6	B6	B6
D8Mit112	08	55.7	B6 FVB	B6	B6 FVB	B6
D8Mit13	08	68.9	B6	B6	B6	B6
D9Mit59	09	0	B6 FVB	B6	B6 FVB	B6
D9Mit229	09	23	B6	B6	B6	B6
D9Mit198	09	43.7	B6	B6	B6	B6
D9Mit82	09	67.8	B6	B6	B6	B6
D10Mit188	10	2.2	B6	B6	B6	B6
D10Mit183	10	12	B6	B6	B6	B6
D10Mit62	10	28.4	B6	B6	B6	B6
D10Mit42	10	41.5	B6	B6	B6	B6
D10Mit95	10	50.3	a190 B6	B6	B6	B6
D10Mit233	10	63.4	B6	B6	B6	B6
D11Mit71	11	0	B6	B6	B6	B6
D11Mit86	11	25.1	B6	B6	B6	B6
D11Mit213	11	53.6	B6 FVB	B6	B6 FVB	B6
D11Mit214	11	77.6	B6	B6	B6	B6
D12Mit109	12	16.4	B6	B6	B6	B6
D12Mit156	12	28.4	B6	B6 FVB	B6 FVB	B6 FVB
D12Mit28	12	47	B6	B6	B6	B6
D12Nds2	12	56.8	B6	B6	B6	B6
D13Mit16	13	-5.3	B6	B6	B6	B6
D13Mit139	13	18.6	B6 FVB	FVB	B6	B6
D13Mit287	13	38.3	B6 FVB	FVB	B6	B6 FVB
D13Mit78	13	59	B6 FVB	B6 FVB	B6	FVB
D14Mit132	14	0	B6	B6	B6	B6
D14Mit141	14	24	B6 FVB	B6	B6	B6 FVB
D14Mit203	14	37.2	B6 FVB	B6 FVB	B6	B6 FVB
D14Mit170	14	69.9	B6 FVB	B6 FVB	B6	B6 FVB
D15Mit174	15	0	B6 FVB	FVB	FVB	FVB
D15Mit183	15	20.8	B6 FVB	FVB	FVB	FVB
D15Mit107	15	41.5	B6 FVB	FVB	FVB	B6 FVB
D15Mit35	15	63.4	B6	B6	B6	B6
D16Mit132	16	0	B6 FVB	B6 FVB	B6	B6 FVB
D16Mit4	16	25.1	FVB	B6 FVB	B6 FVB	B6 FVB
D16Mit189	16	40.4	B6 FVB	B6	B6 FVB	B6
D16Mit71	16	51.4	B6 FVB	B6	B6 FVB	FVB
D17Mit19	17	0	B6	B6	B6	B6
D17Mit180	17	25.1	B6 FVB	B6	B6 FVB	B6
D17Mit93	17	39.3	B6 FVB	B6	B6 FVB	B6
D17Mit123	17	50.3	B6 FVB	B6 FVB	FVB a154	B6 a154
D18Mit19	18	0	FVB	FVB	FVB	FVB
D18Mit149	18	15.3	FVB	FVB	FVB	FVB
D18Mit152	18	25.1				
D18Mit25	18	39.3	B6	B6	B6	B6

D19Mit32	19	0	B6	B6	B6	B6
D19Mit16	19	13.1	B6	B6	B6	B6
D19Mit82	19	25.1	B6	B6 FVB	B6 FVB	B6 FVB
D19Mit35	19	44.8	B6	B6 FVB	B6 FVB	B6
D19Mit76	19	55.7	B6	B6	FVB	B6
total # alleles	192					
% impurity:			24.47916667	24.47916667	28.64583333	24.47916667
% purity:			75.52083333	75.52083333	71.35416667	75.52083333

Mouse #	Genotype
4713	*Atg12fl/fl;RFP+/-;PyMT+;FSP-Cre-*
5337	*Atg12fl/fl;RFP-/-;PyMT+;FSP-Cre-*
5332	*Atg12fl/fl;RFP+/+;PyMT+;FSP-Cre+*
5335	*Atg12fl/fl;RFP+/-;PyMT+;FSP-Cre+*

Genotype	Average Purity
Atg12fl/fl;RFP+/-;PyMT+;FSP-Cre-	75.52083333
Atg12fl/fl;RFP+/+;PyMT+;FSP-Cre+	73.4375

APPENDIX II. Optimized protocol for the isolation of mouse mammary fibroblasts (MMFs)

Materials & Preparations:

Surgery tools, ethanol and materials for necropsy room
DMEM + 10% Calf Serum (NOT heat inactivated) + 1% Penicillin/Streptomycin *(MMF media)*
50 mg/ml gentamicin
Collagenase (Sigma #C5138)
Hyaluronidase (Sigma #H3506)
Scalpels
p60 plates
50 ml conicals
0.22 μm filters
Autoclaved 50 ml jars with sterile magnetic stirbars
Warm waterbath to 37 °C
PBS
Collagen coated plates

Digestion Soln to be made fresh right before use:

(prepare 5 mls digestion soln per MFP)
1.5 mg/ml Collagenase (from 100X stock soln)
125 U/ml Hyaluronidase (from 100X stock soln)
MMF media + 50 μg/ml gentamicin
Filtered through a 0.22 μm filter

Protocol:

1. Sac mice and harvest 3rd and 4th mammary glands (also 5th if you want) using aseptic technique in the necropsy room.
2. Leave mammary glands in PBS on ice while preparing digestion solution (see above)
3. Mince tissue with a sterile scalpel in hood.
4. Add digestion soln and minced tissue to autoclaved 50 ml jars with magnetic stirbars, using ratio above.
5. Incubate in waterbath shaking at 37 °C for 30 min. Digestion is complete when media is cloudy and few tissue chunks remain. A large amount of tissue chunks can be dissociated using an 18G needle/syringe if necessary.
6. Inactivate collagenase by adding 1 uM EDTA.
7. Spin down 1000 rpm 5 min.
8. Resuspend pellet in MMF media + 50 ug/ml gentamicin and plate on a p60 (cells grow better when plated more densely at the start) on collagen coated plates (see collagen coating protocol).

APPENDIX III. Optimized protocol for the adenoviral infection of mouse mammary fibroblasts (MMFs)

Materials & Preparations:

SFM (Serum free MMF media; 0.5% Calf Serum + Penicillin/Streptomycin + 50 ug/ml Gentamicin)
Collagen coated 15 cm plates
MMF media (complete media, for recovery)
Cre-expressing adenovirus (never F/T vials, on the order of 10^{10} PFU/ml)
Bleach container

Protocol:

1. Around 3pm on Day 1, trypsinize and count cells.
2. Spin down at 1000 rpm for 5 min; resuspend at 10^6 cells/ml in SFM
3. Seed 2 x 10^6 cells per coated 15 cm plate in 25 mls of SFM
4. Spike in 24 ul (for 2 x 10^6 cells on this surface area!) of Ad-Cre-GFP virus (viral titer on the order of 4 x 10^{10} PFU/ml).
5. Bleach tips that touch any adenovirus or any pipets that touch adenovirus for at least 30 min (follow standard virus protocol).
6. In the morning of the next day, change cells to complete MMF media (no gentamicin required as cells are out of quarantine now)
7. Check for GFP expression (should be nuclear expression, nearly 100% efficient) within 48 hrs.
8. Check for RFP expression at Day 7.
9. Pellet ~10^5 cells on day of surgery to confirm efficient recombination by western blot.

APPENDIX IV. Optimized protocol for the isolation of mouse cancer associated fibroblasts (CAFs)

Materials & Preparations:

Surgery tools, ethanol and materials for necropsy room
DMEM + 10% Calf Serum (NOT heat inactivated) + 1% Penicillin/Streptomycin *(MMF media)*
PBS + 5% Calf Serum *(FACS Buffer II)*
50 mg/ml gentamicin
Collagenase (Sigma #C5138)
Hyaluronidase (Sigma #H3506)
DNase I (Sigma #D5025)
Red Blood Cell Lysis Buffer (Sigma #R7757)
FcR Blocking Reagent (Miltenyi Biotec #130-092-575)
PDGFRα antibody, PE conjugated (eBiosciences #12-1401)
F4/80 antibody, APC conjugated (eBiosciences #17-4801)
Sterile scalpels
p60 plates
50 ml conicals
0.22 μm filters
70 μm filters
Autoclaved 25 ml jars with sterile magnetic stirbars
Warm waterbath to 37 °C
PBS
Collagen coated plates

Bring to FACS core:
1.5 ml collection tubes (or 15 ml collection tubes for a large sort)
samples on ice
a falcon tube with extra FACS buffer

Digestion Soln to be made fresh right before use:

(prepare 20 mls digestion soln per 5g of tumor tissue or approximate 25 mls digestion soln per genotype/tissue digest)
1.5 mg/ml Collagenase (from 100X stock soln)
125 U/ml Hyaluronidase (from 100X stock soln)
MMF media + 50 μg/ml gentamicin
Filtered through a 0.22 μm filter

Protocol:

1. Sac mice and harvest tumors using aseptic technique in the necropsy room.
2. Weigh tumor burdened mammary glands.
3. Leave mammary glands in PBS on ice while preparing digestion solution (see above).
4. Mince tissue with a sterile scalpel in hood.
5. Add digestion soln and minced tissue to autoclaved 25 ml jars with magnetic stirbars, using ratio above or noted approximation.
6. Incubate in waterbath shaking at 37 °C for 20-30 min. Digestion is complete when media is cloudy and few tissue chunks remain.
7. Inactivate collagenase by adding 1 uM EDTA.
8. Transfer digested tissue to a 50 ml falcon tube. Rinse the autoclaved jar with 12.5 mls media and add to falcon.
9. Spin down 1000 rpm 5 min.
10. Perform an RBC lysis: Incubate the pellet for 1 min at RT in 1ml of RBC Lysis Buffer.
11. Immediately add 20 mls of PBS
12. Spin down 1140 rpm 7 min

13. Repeat steps 12 and 13 if pellet looks very bloody.
14. Resuspend in 5 mls MMF media. Vortex to thoroughly resuspend the pellet.
15. Incubate with 100 ug/ml DNase, 2 min in 37 °C waterbath (for 500 ug/ml aliquot stock, this is 10 ul). Vortex after 5 min. If after 10 min of DNase treatment pipetting is still difficult, repeat this step.
16. Remove large, undigested tissue chunks with a 30 μm filter *(rinse filter to wet with media first before applying cell suspension)*. Wash filter and falcon tube with media before discarding to retrieve any cells.
17. Count cells (you may want a dilute suspension ~50 mls) During any downtime, keep cells on ice.
18. Perform FcR block by resuspending cells up to 10^7 per 90 ul FACS Buffer II and adding 10 ul FcR Blocking Reagent *(1:10 dilution of antibody)*. When working with fewer than 10^7 cells, use the same volume. For more than 10^7 cells, scale up reagent volume and total volumes accordingly.
19. Allocate cell suspension to 5ml polypropylene FACS tubes and prepare essential staining control tubes (keeping cells on ice); ~500 ul per tube or less for total volume.
    a. 100-500 ul of unstained cell suspension
    b. 100-500 ul of isotype cell suspension (rat IgG2a for both)
    c. 100-500 ul of PDGRFα-PE alone
    d. 100-500 ul of F4/80-APC alone
20. Add antibodies:
    a. 1:50 dilution of PDGRFα-PE
    b. 1:50 dilution of F4/80-APC
21. Incubate on ice for 30 min in the dark.
22. Wash cells with at least 1 ml FACS Buffer II per tube.
23. Spin down 1000 rpm 5 min
24. Resuspend pellet in 500 ul FACS Buffer II.
25. Keep cells on ice and head to FACS sorter. Remember to bring 15 ml collection tubes with at least 5 ml MMF media to retrieve sorted cells, as well as extra FACS Buffer II for dilution.
26. Spin down sorted cells 1000 rpm 5 min.
27. Resuspend in MMF media and plate on a p30 (cells grow better when plated more densely at the start) on collagen coated plates (see collagen coating protocol).

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